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# Identification of three *cry 2A* genes in an indigenous *Bacillus thuringiensis* strain, 67-3

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**ABSTRACT:**
The Cry2A proteins of *B. thuringiensis* are promising candidates for the management of resistance development in insects due to its differences from the currently used Cry1A proteins, in structure and mode of action. The universal forward primer (UniF), reverse primers specific to *cry2Aa*, *cry2Ab* and *cry2Ac* (2AaR, 2AbR and 2AcR) were used to detect the presence of above three *cry2A* genes in an indigenous isolate of Bt, 67-3 by PCR. Nucleotide sequence data obtained from the amplified DNA fragments confirmed the presence of *cry2Aa*, *cry2Ab* and *cry2Ac* genes in the indigenous isolate of Bt, 67-3. None of the 16 reference strains and 126 isolates of Bt screened by Ben-Dov *et al* [1] contained more than two *cry2A* genes. Further cloning and sequencing of the *cry2A* genes of the indigenous Bt strain 67-3 may yield new genes of the respective subgroups.

**Key words:** *Bacillus thuringiensis*, indigenous strain, *cry2A* genes, gene profiling.

## INTRODUCTION

*Bacillus thuringiensis* (Bt), is a well-known gram-positive, spore-forming soil bacterium that forms parasporal insecticidal crystal proteins during the stationary and sporulation phase of its growth cycle. These proteins are termed delta-endotoxins because of their intercellular location and have been used for many years as successful biological insecticides [2]. Molecular potency of Bt toxins is high compared to the chemical pesticides, *i.e*., 300 times higher than synthetic pyrethroids and 80,000 times stronger than organophosphates [3]. Cloning of the first crystal protein gene (*cry*) of Bt was reported [4], since then more than 270 *cry* genes have been cloned, characterized, and their classification based on amino acid sequence similarity of their proteins [5]. The commercial use of Bt as suspension of spores and inclusions has been limited in part due to the need to spray at rather frequent intervals, in order to sustain an effective level of biopesticide. This problem has been circumvented by engineering plants (cotton, corn *etc*.) to produce the Bt toxin. Therefore, the transgenic crop that produces a potent toxin such as Cry1Ac can control some key lepidopteron pests. The first version of Bt - cotton, Bollgard®I, developed by the multinational company, Monsanto, expresses Cry1Ac and mainly targets *Heliothis virescens*, the major pest of cotton in USA. Transgenic Bt-cotton expressing Cry1Ac has been registered for commercial cultivation in India during the year 2002 and it primarily targets *H. armigera*. However, continued use of a single Bt protein in Bt-cotton will lead to resistance development in lepidopteran insects. The Cry2A proteins of Bt are promising candidates for management of resistance development in insects due to its differences from the currently used Cry1A proteins, in structure [6] and mode of action [7]. Routine replacement of *cry* genes or pyramiding of *cry* genes could be useful for effective control of insect pests by Bt transgenic plants. The combination of *cry2Ab* and *cry1Ac* gene in Bollgard® II cotton have been permitted for commercial use in the USA and it has been shown that Bollgard® II provides superior control of lepidopteran pest and is expected to have positive implication for resistance management especially with respect to cotton bollworms. But the Indian populations of *H. armigera* were thirty-five fold less susceptible to Cry2Aa than Cry1Ac [8]. Studies are insufficient on susceptibility of Indian populations of *H. armigera* to different Cry2A proteins. Significant sequence similarity exists among the *cry* genes of Bt, however, individual gene products vary in their levels and spectra of toxicity to different insects [9]. In this context, it is appropriate to clone and characterize *cry2A* genes from new isolates of Bt which could encode more toxic Cry2Ab proteins due to variation in their sequences. Screening of Bt strains for their *cry* gene content forms the basis to clone the desired genes. The Polymerase Chain Reaction (PCR) is a molecular tool widely used to screen Bt strain collections for their *cry* genes content [1, 10, 11, 12 and 13]. The efficacy of PCR in identifying the large family of *cry* genes, with amino acid identities ranging from less than 45 per cent to more than 95 per cent, is based on the presence of conserved regions. For practical reasons, primer pairs designed from highly conserved regions and recognizing entire *cry* gene sub family are often used in a preliminary screening prior to performing a second PCR with gene specific primers. An enhanced strategy using PCR for rapid screening of Bt strains to detect three *cry2A* genes by a pair of universal primers (Uni2F & Uni2R). At that time nucleotide sequence data were known for three *cry2A* genes only, *viz*., *cry2Aa*, *cry2Ab* and *cry2Ac*. So, three more reverse primers (2AaR, 2AbR and 2AcR) specific to *cry2Aa*, *cry2Ab* and *cry2Ac* were also reported [1].

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**Table 1: Primers used for screening of *cry2A* genes of Bt [1]**

| SL. No. | Primer name | Sequence (5'→ 3') | Position from ATG | Gene recognized | Size of the amplicon (bp) | Annealing temprature |
|---|---|---|---|---|---|---|
| 1. | UniF | GTTATTCTTAATGCAGATGAATGGG | 571-595 (*cry2Aa1)* | *cry2Aa* | 498 | 58º C |
| 2 | 2AaR | GAGATTAGTCGCCCCTATGAG | 1067-1048 (*cry2Aa1)* | | | |
| 3 | UniF | GTTATTCTTAATGCAGATGAATGGG | 571-595 (*cry2Ab1*) | *cry2Ab* | 546 | 64º C |
| 4 | 2AbR | TGGCGTTAATGGGGGGAGAAAT | 1116-1093 (*cry2Ab1*) | | | |
| 5 | UniF | GTTATTCTTAATGCAGATGAATGGG | 571-595 (*cry2Ac1)* | *cry2Ac* | 724 | 66º C |
| 6 | 2AcR | GCGTTGCTAATAGTCCCAACAACA | 1294-1272 (*cry2Ac1)* | | | |

## MATERIALS AND METHODS

### GENOMIC DNA ISOLATION AND AMPLIFICATION BY PCR

Genomic DNA of an indigenous Bt strain 67-3 was isolated and used as a template for polymerase chain reaction (PCR) amplification. The universal primer (uni F) and the respective reverse primer for *cry2Aa*, *cry2Ab* and *cry2Ac* were used. The primers details are listed in Table.1. Polymerase chain reaction (PCR) for the DNA fragment was carried out with a Taq DNA polymerase (Bangalore Genei Pvt.Ltd., India) in 40 µl reaction volume. Each 40 µl reaction mixture contained 100 ng of genomic DNA of *Bacillus thuringiensis* strain 67-3 ,50-100 ng of forward and reverse primers, each dNTP at a final concentration of 200-300 µM, and 2.5 U of Taq polymerase in 1X Taq buffer (with 15 µM $Mgcl_2$). The PCR was performed for 30 cycles as follows: 94 °C for 1 min, 60 °C for 45 sec and 72 °C for 1.0 min., the final extension was performed for 7 min at 72 °C. The amplicons were column purified and examined on 1.2 % agarose gel.

### RECOMBINANT DNA PROCEDURES

Restriction digestion and ligation reactions were performed out as per manufacture instruction. Agarose gel electrophoresis, *E.coli* competent cells and their transformation were done according to the standard procedure.

### RESULTS AND DISCUSSION

In the present study, a reverse primer (2AaR, 2AbR and 2AcR) specific to *cry2Aa, cry2Ab* and *cry2Ac* genes were designed. Genomic DNA of an indigenous Bt strain, 67-3 was isolated and screened by PCR with *cry2A* gene specific screening primers. Amplification of DNA fragments of expected size in all the three cases (~500, 550 and 700bp for *cry2Aa, cry2Ab* and *cry2Ac* respectively) indicated presence of the above three *cry2A* genes in Bt strain, 67-3 (Figure 1).

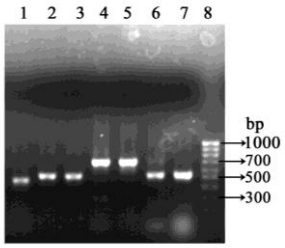


**Figure 1**
Agarose gel electrophoresis of DNA fragments amplified from Bt strain, 67-3, by PCR with the primers specific to three different *cry2A* genes.
Lane 1, *cry2Aa*; Lanes 2-3, *cry2Ab*; Lanes 4-5, *cry2Ac*;Lanes 6-7, *cry2Ab*; Lane 8, 100bp ladder.

To confirm presence of all the three *cry2A* genes, the amplified DNA fragments were cloned by using insT/Aclone pcr cloning kit. The recombinant clones were selected on LB agar plates containing X-Gal, IPTG and ampicillin. Presence of insert DNA in the recombinant clones was confirmed by colony PCR with uniF and specific reverse primers as well as by restriction digestion of the recombinant plasmids with *Xba*I and *Pst*I. The recombinant clones were sequenced with M13F and M13R primers through automated sequencing (Bangalore Genei). The sequence data obtained from the recombinant plasmids contained the internal region of the respective *cry2A* genes along with vector sequences at the ends. These results confirmed the presence of *cry2Aa, cry2Ab* and *cry2Ac* [Gen Bank accession No. EU360896 (*cry2Ac*)] in the indigenous isolate of Bt, 67-3. The blast analysis of the nucleotide sequences amplified from the indigenous Bt strain 67-3, showed 100 per cent similarity to the corresponding regions of the holotype sequences of the *cry2Aa* and *cry2Ab* genes (Data not

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shown). BLAST analysis revealed that the nucleotide sequence data obtained from the *cry2Ac* gene of Bt strain, 67-3 showed 98 percent homology with that of *cry2Ac1* (Acc. No. X57252). Deduced amino acid sequence of Cry2Ac protein of Bt strain, 67-3 is shown in Fig. 2. The deduced amino acid sequence of the recombinant Cry2Ac protein (623 amino acids) varies at 13 positions from its holotype (Cry2Ac1).

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1
MNTVLNNGRNTTCHAHNVVAHDPFSFEHKSLNT
IEKEWKEWKRTDHSLYVAPIVGTVGSFLLKKVG
SLVGKRILSELQNLIFPSGSIDLMQEILRATEQ
FINQRLNADTLGRVNAELAGLQANVAEFNRQVD
NFLNPNQNPVPLAIIDSVNTLQQLFLSRLPQFQ
IQGYQLLLLPLFAQAANLHLSFIRDVILNADEW
GISAATVRTYRDHLRNFTRDYSNYCINTYQTAF
RGLNHRLPDMLEFRTYMFLNVFEYVSIWSLFKY
QSLLVSSGANLYASGSGPTQSFTAQNWPFLYSL
FQVNSNYVLNGLSGARTTITFPNIGGLPGSTTT
QTLHFARINYRGGVSSSRIGQANLNQNFNISTL
FNPLQTPFIRSWLDSGTDREGVATSTNWQSGAF
ETTLLRFSIFSARGNSNFFPDYFIRNISGVVGT
ISNADLARPLHFNEIRDIGTTAVASLVTVHNRK
NNIYDTHENGTMIHLAPNDYTGFTVSPIHATQV
NNQIRTFISEKYGNQGDSLRFELSNTTARYTLR
GNGNSYNLYLRVSSIGSSTIRVTINGRVYTANV
NTTTNNDGVLDNGARFSDINIGNVVASANTNVP
LDIQVTFNGNPQFELMNIMFVPTNLPPLY 623
```

**Note:** Amino acid residues of Cry2Ac varied from its holotype Cry2Ac1 is indicated in bold faces

**Figure 2. Deduced amino acid sequence of Cry2Ac protein**

Earlier study of Ben-Dov *et al* [1] showed presence of a single *cry2A* gene in one of 16 reference and 26 of 126 new isolates of Bt. Presence of two *cry2A* genes were observed in 4 of the 16 reference and 35 of the 126 new isolates of Bt. None of the 16 reference strains and the 126 isolates of Bt contained more than two *cry2A* genes [1]. Variation of a single amino acid can significantly influence the level of toxicity in Cry proteins [15, 16]. As per the recent nomenclature, the *cry* genes whose products are different in amino acid sequence but are more than 95 per cent identical to each other are given separate quaternary ranks by Arabic number at the end (eg. *cry1Aa1, cry1Aa2).* Therefore, cloning and sequencing of the *cry2A* genes of the indigenous Bt strain 67-3 may yield new genes of the respective subgroups.

## CONCLUSION

The Cry2A proteins of *Bacillus thuringiensis* (Bt) are promising candidates for management of resistance development in insects due to their differences from the currently used Cry1 proteins, in structure and insecticidal mechanisms. Since India is very rich in biodiversity and genetic resources, different *B. thuringiensis* strains available in the country are valuable source for identification of indigenous, novel *cry* genes, which could be used for the control of insect pests of crop plants. The new variants of the *cry* gene subgroups and chimeric *cry* gene sequences could encode crystal proteins with significant difference in toxicity due to variation in their sequences. Cloning of new *cry* genes and construction of chimeric Cry proteins through recombinant DNA technology can be an efficient strategy to enhance the level of toxicity for effective control of the target pest.

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